Hum Genet (1996) 97:770–776

ORIGINAL INVESTIGATION

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Detection of a germline mutation and somatic homozygous loss of the von Hippel-Lindau tumor-suppressor gene in a family with a de novo mutation

A combined genetic study, including cytogenetics, PCR/SSCP, FISH, and CGH

Received: 15 July 1995 / Revised: 20 October 1995

Abstract von Hippel-Lindau (VHL) disease is a pleiotropic disorder featuring a variety of malignant and benign tumors of the eye, central nervous system, kidney, and adrenal gland. Recently the VHL gene has been identified in the chromosomal region 3p25-26. Prognosis and successful management of VHL patients and their descendants depend on unambiguous diagnosis. Due to recurrent hemangioblastomas, a 29-year-old patient without familial history of VHL disease was diagnosed to be at risk for the disease. Histopathological examination of a small renal mass identified a clear cell tumor with a G1 grading. Genetic characterization of the germline and of the renal tumor was performed. Polymerase chain reaction/single strand conformation polymorphism (PCR/SSCP) analysis with primers from the VHL gene identified a deletion of a single nucleotide in exon 2 in the patient's germline and in the tumor, but not in the DNA of his parents. This deletion therefore must be a de novo mutation. Comparative genome hybridization (CGH) and fluorescence in situ hybridization (FISH) analysis of the G1 tumor with differentially labelled yeast artifical chromosome (YAC) clones showed loss of 3p and of the 3p26 signals, respectively. In conclusion, we identified a de novo germline mutation in the VHL gene of a young patient and a somatic chromosome 3p loss at the homologous chromosome 3 in his renal tumor. Our results suggest a recessive mode of inactivation of the VHL gene, providing solid evidence for its tumor-suppressor gene characteristics. Our data show the diagnostic potential of genetic testing, especially in patients without VHL family history. Furthermore, the findings of homozygous inactivation of the VHL gene in a G1 tumor support the notion that the inactivation of the VHL gene is an early event in tumorigenesis of renal cell carcinoma.

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Introduction

Von Hippel-Lindau (VHL) disease is an autosomal dominant inherited disorder with virtually complete penetrance and a highly variable expressivity. The incidence of VHL is approximately 1:36 000, though it is possible that there are regional variations (Maher et al. 1991b; Maher 1993). Characteristic and frequently observed lesions in VHL are renal cell carcinomas (RCCs), angiomatosis retinae (AR), cerebellar and spinal hemangioblastomas (CH), pheochromocytomas (pheo), and multiple cysts in parenchymal organs. The minimum criteria of the disease are defined either by the occurrence of two of the major features (AR and CH) in a patient without a familial history of VHL or by one feature (AR, CH, RCC or pheo) and a family history (Melmon and Rosen 1964; Horton et al. 1976; Neumann 1987; Maher et al. 1991b). By means of their clinical characteristics, only about half of the patients will be diagnosed as gene carriers by the age of 25 years. As all VHL-associated tumors also occur in a sporadic form, due to the variable expressivity, clinical diagnosis of VHL might be very difficult (Glenn et al.1991, 1992; Seizinger et al. 1991; Davies et al. 1994). This holds especially true for a de novo diagnosis. New germline mutations might

occur in about 5% of all VHL families (Neumann et al. 1995). Mutations in the VHL gene have been found in 39–73% of VHL families (Crossey et al. 1994b; Richards et al. 1994; Whaley et al. 1994). This progress in molecular genetics provided the possibility for genetic testing in patients at risk. Genetic testing is of special clinical significance for patients with VHL manifestations and absence of family history.

One of the most serious and life-threatening complications in VHL are RCCs, which will be seen in 17–55% of affected gene carriers. Compared to their sporadic counterparts, RCCs in VHL patients occur at an earlier age (45 versus 62 years) and are frequently bilateral, multifocal, and associated with renal cysts. After onset, RCCs in VHL may show a slower growth rate and metastasize later than the sporadic forms (Choyke et al. 1995).

Predisposition to cancer is the hallmark of families with germline mutations in a tumor-suppressor gene (Knudson 1971). Somatic loss of the balancing wild-type allele by different mechanisms is the mode of tumorigenesis in these conditions (Cavenee et al. 1983; Kovacs and Kung 1991). The VHL gene has been supposed to be a tumor-suppressor gene (Seizinger et al. 1988; Decker et al. 1989; Tory et al. 1989). Recently, the gene has been isolated (Latif et al. 1993) and actually found to function as a tumor suppressor (Crossey et al. 1994a; Gnarra et al. 1994; Shuin et al. 1994). The gene has been located in 3p25–26 by genetic linkage analysis (Seizinger et al. 1988, 1991; Hosoe et al. 1990; Vance et al. 1990; Maher et al. 1991a;) and physical mapping (Latif et al. 1993; Decker et al. 1994). The biological function of the VHL gene product is unknown[1].

Mutations of the VHL gene have been identified in 33–57% of sporadic renal cell carcinomas (Foster et al. 1994b; Gnarra et al. 1994; Shuin et al. 1994; Whaley et al. 1994). These mutations have been seen in early as well as in advanced forms, suggesting a critical role of the VHL gene for the origin of this malignancy. Thus the finding of VHL mutations in renal tumors may allow prediction of the malignant potential of small tumors, the cytological staging of which may not be conclusive.

All RCCs in VHL described so far have been of the clear cell type. To distinguish between a renal adenoma and a "true" RCC of small size might be difficult. The Bell's rule of tumor size as a criterion for malignancy has often been questioned, but tumor size is still a helpful criterion, especially in a case of an uncertain tumor grading (e.g., tumor of uncertain dignity). There is a need for a refined evaluation of the malignant potential in small renal tumors. This could be overcome by characterization of genetic alteration.

In this study we wanted to show the applicability of the polymerase chain reaction/single-strand conformation polymorphism (PCR/SSCP) approach for detecting a de novo germline mutation in a patient without family history of VHL. Furthermore, we studied the somatic alterations of the patient's renal cell tumor to search for homozygous inactivation of the VHL gene.

[1] Compare two articles: Science (1995) 269:1402 and Science (1995) 269:1444 published after submission of this manuscript

Materials and methods

Case report

A 29-year-old man presented with recurrent spinal hemangioblastomas (Fig. 1). Due to the early onset and recurrence of the disease, a refined clinical work-up was performed including abdominal and ophthalmological exploration. The eye examination revealed an ambiguous result without the clear picture of an AR. Ultrasonography and computed tomography showed pancreatic and renal cysts and a renal mass of 19 mm (Fig. 2). Therefore the patient was classified as a prime candidate suspicious of von Hippel-Lindau disease. The tumor was removed, sparing the kidney. The patient recovered from surgery without any problems. In the patient's family no other members presented with any signs of VHL disease. The patient's aunt died from breast cancer at age 46 years, the grandfather died of leukemia at age 56 years. Informed consent was obtained from all individuals tested in this study.

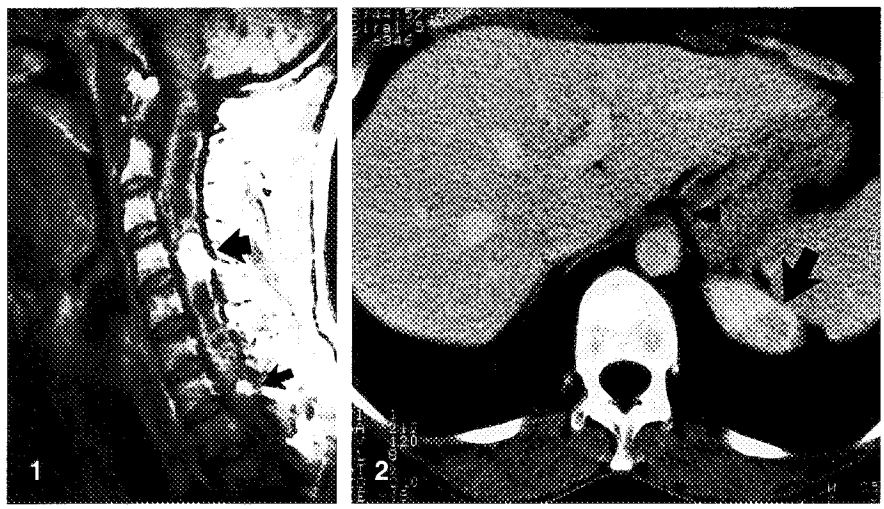


Fig. 1 Sagittal T1-weighted magnetic resonance imaging of the patient's cervical spinal cord. The *arrow* indicates the spinal hemangioblastoma

Fig. 2 Computed tomography scan of the abdomen without contrast showing the small renal mass (*arrow*)

Histopathological examination

Both formaldehyde-fixed paraffin-embedded and fresh-frozen sections of the renal tumor were studied. All specimens were stained with hematoxylin and eosin (H and E) using standard techniques.

Karyotyping

Blood was drawn for cytogenetic studies. Tumor specimens were aseptically dissaggregated and cultured for several days. Chromosome analysis on lymphocytes and tumor cells were performed according to established methods (Decker et al. 1990).

SSCP

DNA was isolated from blood and tumor cells as reported. PCR was performed applying the primers from exons 2 and 3 of the VHL gene (Crossey et al. 1994a; Foster et al. 1994a). The amplification was performed using a Trioblock (Biometra, D-3400 Göttingen, Germany). The conditions were as follows: initial denaturation 4 min 96° C; 35 cycles of denaturing 40 s 96 °C, annealing for 40 s 65° C, extension for 40 s at 72 °C; final extension for 4 min at 72° C. The PCR products were separated in a 8% polyacrylamide gel with 2% glycerine, at 8° C and 15° C using the TGGE system (Diagen, D-40724 Hilden, Germany). Voltage was 200 V (30 mA) for 3 and 6 h . Gels were silver-stained according to standard methods.

DNA sequencing

DNA sequencing was performed on an automated DNA sequencer (ABI 373A, Applied Biosystem, USA) applying the manufacture's sequencing kit and the primer set for exons 2 and 3 (Gnarra et al. 1994; Richards et al. 1994; Shuin et al. 1994).

Comparative genome hybridization

DNA was extracted as described (Decker et al. 1989). Genomic DNA prepared from the tumor specimen and normal tissue was differentially labelled with biotin and digoxigenin, respectively. The labelled DNAs were mixed equimolarly and applied for chromosomal in situ hybridization (CISS) to normal chromosome spreads. The hybridization and the detection using fluorescein isothiocyanate (FITC) and tetraethylrhodamine (TRIC), respectively, was performed applying recently published methods (Kallioniemi et al. 1992; Ried et al. 1994). For image acquisition, an epifluorescence microscope (Zeiss Axiophot, Germany) was used equipped with a cooled charge coupled camera (CCD; KAF 1400, Photometrics, Tucson, Ariz.; 1400 chip). The analysis was performed on an Apple Quadra computer (Macintosh, USA) applying established software (du Manoir et al. 1993).

Fluorescence in situ hybridization

The same cytogenetic preparations were used for fluorescence in situ hybridization (FISH) and for chromosome studies. The probes used were yeast artificial chromosome (YAC) clones derived from the chromosomal regions of 3p25 and chromosome 3qter. They were gifts from Dr. Riethman, The Wistar Institute, Philadelphia, USA (Lengauer et al. 1992), and from Dr. Helen Donis-Keller, Division of Human Molecular Genetics, Washington University, School of Medicine, St. Louis, USA, respectively. Labelling was performed by nick-translation according to established methods (Ried et al. 1993). The fluorochromes were FITC (yellow) for 3p26 and TRIC (red) for the 3qter probe. Analysis was performed on an epifluorescence microscope (Zeiss Axiophot, Germany) with a CCD camera.

Results

Histopathological examination

Macroscopic examination revealed a small (1.3 cm diameter) lobulated solid tumor mass with a yellow cut sur-

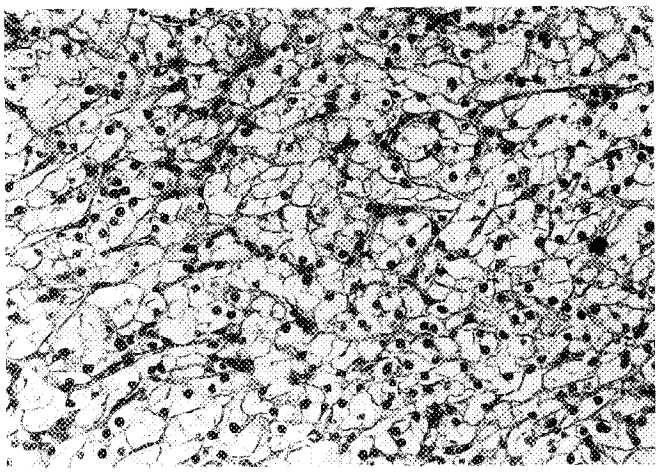


Fig. 3 Histopathology of the renal tumor showing a G1 tumor of clear cell type. (Hematoxylin and eosin staining, original magnification × 500)

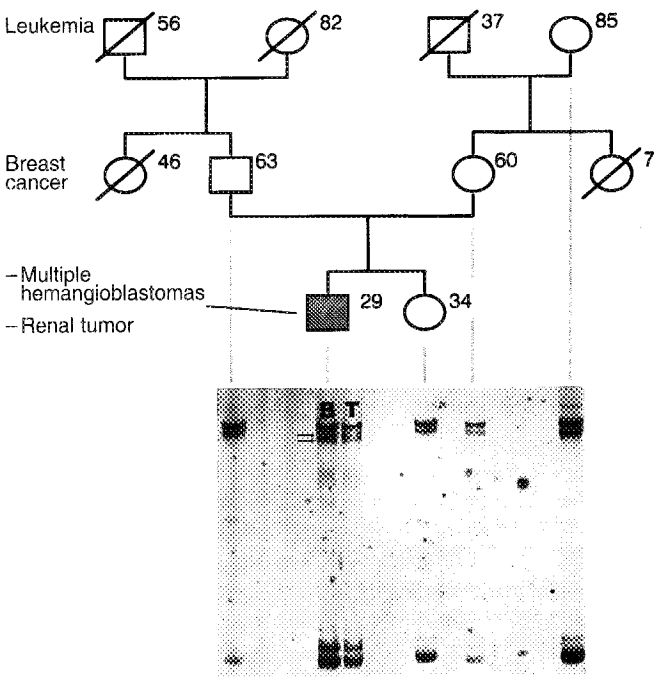
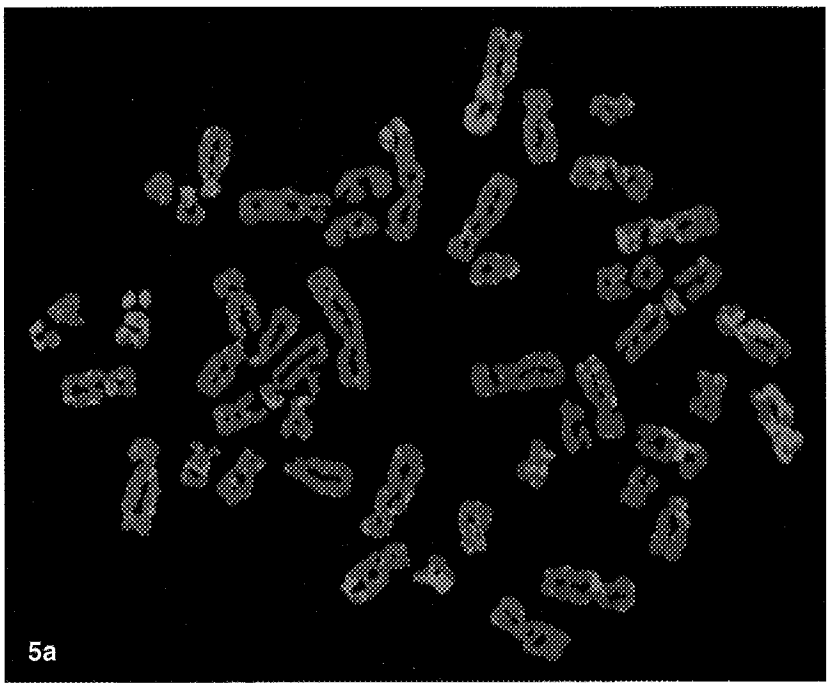
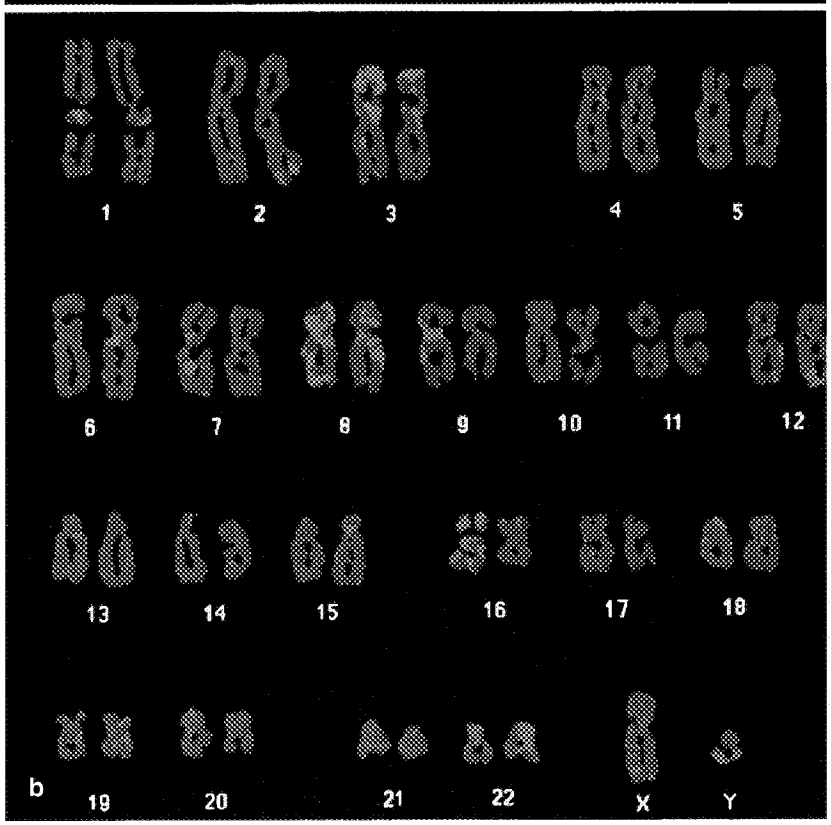
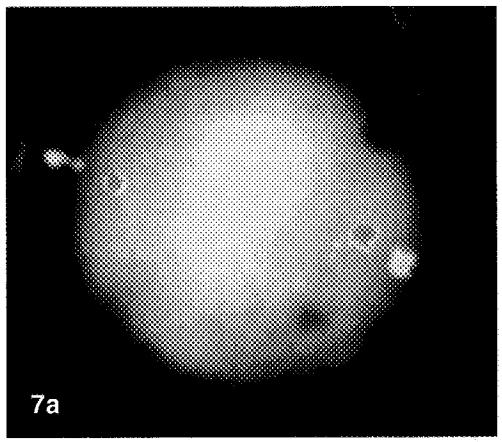


Fig. 4 Patient's pedigree and polymerase chain reaction/single-strand conformation polymorphism (PCR/SSCP) analysis with primers from exon 2 of the von Hippel-Lindau (VHL) gene. The patient (29 years) was the only family member showing symptoms of VHL disease. The SSCP gel showed a mobility shift resulting in an additional band (*small arrows*) in the blood (*B*) as well as in the tumor (*T*) DNA. This altered mobility pattern was also seen in the DNA heteroduplices (bands below). Sequencing revealed a deletion of a thymidine from exon 2 of the VHL gene. All other family members tested, including his 34-year-old sister, did not show any alteration of the gene, either by SSCP analysis or by sequencing







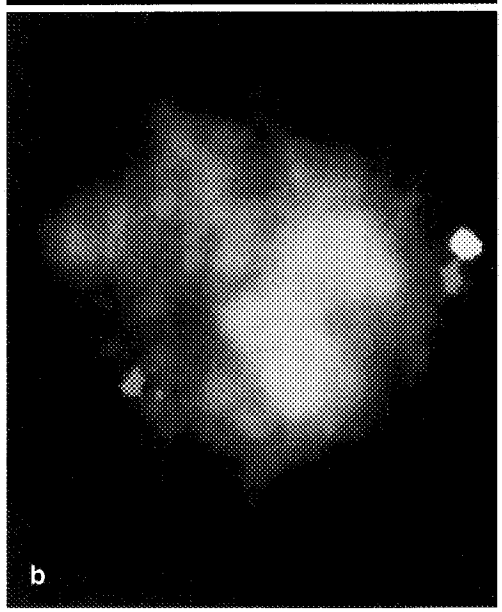


Fig. 5 Representative metaphase spread (**a**) and karyogram (**b**) indicating the fluorescence ratio values measured for the individual chromosomes. Chromosome 3p and chromosome 8 appeared to be underrepresented (compare with Fig. 6)

Fig. 7 Fluorescence in situ hybridization experiments on a normal (**a**) and a tumor (**b**) cell of the patient with a germline mutation in the VHL gene. The 3p25 yeast artificial chromosome (YAC) clone is detected by FITC (yellow/green), the chromosome 3q YAC clone with TRIC (red). In the tumor cell, loss of one 3p25 signal was seen

face. Light microscopically (Fig. 3) the tumor was composed of solid sheets of transparent or clear cells with small condensed hyperchromatic nuclei (= grade 1). The cytoplasm was filled with lipid droplets and glycogen deposits. There was no pronounced mitotic activity. The resection margins were free of tumor. The surrounding parenchyma was compressed like a pseudocapsule.

Germline study

Figure 4 shows the pedigree of the patient at risk for VHL. No other family member presented with typical symptoms for VHL. High resolution banding karyotyping of normal lymphocytes of the patient did not reveal any chromosomal alterations. Molecular studies were performed on the constitutional DNA of those family members from whom blood was available. SSCP analysis of exon 2 (Fig. 4) showed an altered banding pattern only for the patient's blood and tumor DNA. The finding of a mutated VHL gene could be confirmed by DNA sequencing. Sequencing was repeated several times and performed on both, the sense and anti-sense strands, revealing the same mutation for both the constitutional and the tumor DNA. One nucleotide was deleted from the pentameric thymidines nucleotides 653–657 of exon 2, predicted to result in a stop codon. None of the other family members showed an altered pattern in SSCP analysis. Sequencing of constitutional DNA from all of them revealed the normal wild-type sequence of the VHL gene (Genbank accession number L15409). Therefore the mutation seen in our patient represents a de novo mutation.

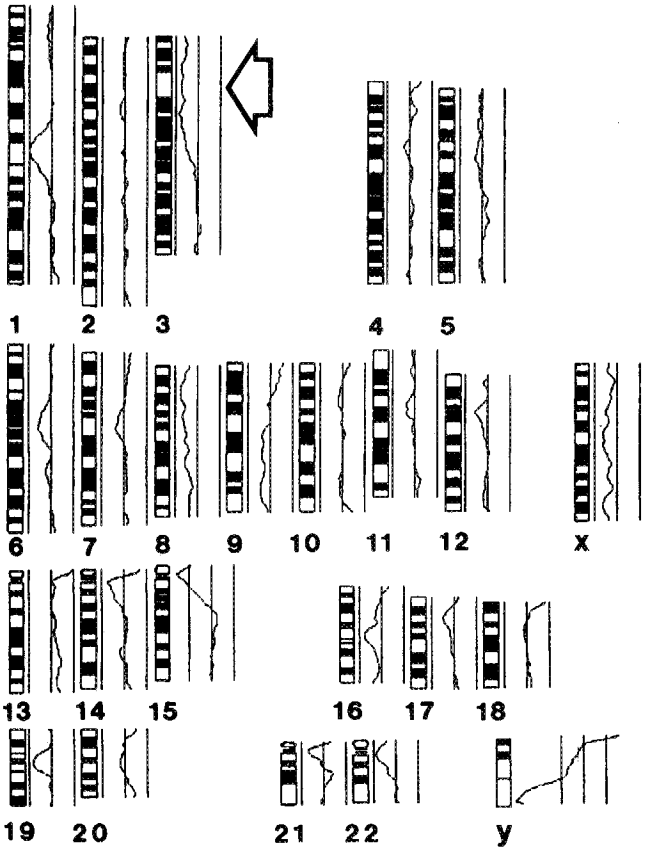


Fig. 6 Chromosome idiograms with ratio profils summarizing the results from ten chromosome metaphases analyzed. The ratio of fluorescein isothiocyanate/tetraethylrhodamine (FITC/TRIC) fluorescence intensities for each chromosome or chromosomal region reflects the relative copy number in the tumor genome compared to that of the normal kidney tissue of the patient. The three lanes placed at the right side of the idiograms indicate the ratios: 0.5 (*left*) for monosomies (1N), 1 (*middle*) for disomies (2N), and 1.5 (*right*) for trisomies. The chromosomal regions 3p distal to 3p11-p14 and 8(q24→pter) are indicated to be underrepresented in the tumor

Somatic alterations

Tumor cells were cultured and several attempts of harvesting for chromosome analysis were made. Due to the low growth rate, sufficient metaphase spreads could not be achieved. Therefore a method which allows cytogenetic examination irrespective of mitotic activity was used. The tumor genomic DNA was compared with normal tissue DNA applying comparative genome hybridization (CGH). Figure 5 shows a typical ratio image of a metaphase spread indicating underrepresentation of the short arm of chromosome 3 distal to 3p11–14. The mean of the ratio profile calculation of ten chromosomes analyzed is seen in Fig. 6. An extended deletion indicated loss of one homolog of the chromosomal segment distal to 3p11–14. This region includes the VHL region in 3p25–26. To confirm these results we performed interphase cytogenetics applying FISH. As probes, YAC clones from the chromosomal region of the VHL gene (e.g., 3p25–26), and 3qter were used and differentially labelled (e.g., yellow and red, respectively). Figure 7 shows a typical result of the hybridization experiments. The constitutional interphase nucleus of a lymphocyte (Fig 7a) revealed two signals for both the 3p25 and the chromosome 3qter probe. The tumor nucleus (Fig 7b) showed two signals for the probe from 3qter, but only one signal for the 3p25 region, indicating loss of this region in one homolog. In 25 out of 30 cells analyzed, the same loss was seen. SSCP analysis of the tumor DNA showed the same mutation as the constitutional DNA, suggesting the presence of the disease allele. The results of FISH analysis of the tumor nuclei showed loss of the chromosomal region containing the VHL gene in one chromosome 3. Since the germline mutation was still present in the tumor, the chromosomal deletion affected the wild-type chromosome.

Discussion

De novo diagnosis presents a difficulty in clinical diagnosis of VHL disease since a major criterion, that of family history, is missing. Since VHL disease is characterized by various complications involving many different organs, late, false or missed diagnosis may result in a severe course of the disease and premature death. Since clinical diagnosis depends on the onset of symptoms that may be age-dependent, gene carriers will not be identified before they suffer from complications. Among those patients without a family history, tumors could be mistaken to be of sporadic origin. These limitations can be overcome by molecular testing for specific germline mutations, which has become feasible (Crossey et al. 1994b) since the gene has been cloned (Latif et al. 1993).

We describe the identification of a de novo VHL germline mutation by PCR/SSCP analysis in a patient with hemangioblastomas and a RCC. The young age of 29 years

and multiple tumors put him into the risk category of individuals affected by a hereditary disorder. The combination of recurrent hemangioblastomas and renal clear cell carcinoma suggested his being at risk of having VHL disease.

We confirmed these assumptions by the identification of a single nucleotide deletion within the VHL gene, which is predicted to result in a premature stop codon that will result in a truncated VHL protein. This mutation was absent in the parents' DNA. Our results encourage the applicability of the PCR/SSCP approach in the detection of VHL germline mutations in patients with uncertain diagnosis. Despite the drawback of a moderate sensitvity of 85–90%, depending on the length of the PCR products, the sequence context and the amplification conditions, and the fact that only about 75% of the VHL germline mutations can be detected (Crossey et al. 1994b), we were able to score a single-base deletion that identified our patient as a VHL gene carrier. We suggest that the PCR/SSCP approach will not only be helpful in following the segregation pattern in established affected pedigrees but, furthermore, provides a powerful diagnostic tool in patients without an established disease history.

The VHL gene has been speculated (Seizinger et al. 1988; Decker et al. 1989; Tory et al. 1989) and shown (Maher et al.1991b; Latif et al.1993; Maher 1993) to function as a tumor-suppressor gene. Its involvement, even in non-VHL-associated tumors, has been shown (Maher et al. 1991b; Latif et al. 1993; Maher 1993; Kanno et al. 1994). In this study we report a somatic alteration in a renal tumor from a patient with a de novo diagnosis of VHL. Conservative surgery was performed (Steinbach et al. 1992). The tumor was small in size (< 2 cm) and exhibited the histopathological picture of a highly differentiated G1 renal clear cell tumor. The molecular analysis of the germline and the tumor in conjunction with the CGH and FISH data indicated homozygous loss of the VHL gene in the renal tumor. As tumor morphology did not allow unequivocal classification of this tumor as an adenoma with respect to dignity, one might speculate about the indication of the malignant potentials by the VHL mutation. On the other hand, there are also benign VHL-associated tumors such as pheochromocytomas (C. Neuhaus et al., unpublished data) and hemangioblastomas (Kanno et al. 1994), which exhibit somatic molecular involvement of the VHL gene. Therefore homozygous loss of the VHL gene is not a definite criterion for malignant progression. Nevertheless, somatic homozygous inactivation – a major feature of a tumor-suppressor gene – has been detected in association with an early stage of renal tumor formation in our de novo case. VHL alterations could either be one of the prerequisites for malignant transformation, as a high percentage of sporadic RCCs tested (Maher et al. 1991b; Maher 1993; Foster et al. 1994b; Gnarra et al. 1994; Shuin et al. 1994) showed VHL involvement regardless of their staging or grading, or it could be the determining factor for tissue specificity. Further investigations on other non-VHL-associated highly malignant tumors with 3p alterations such as small cell lung cancers or mesotheliomas (Gnarra et al. 1994; Sekido et al. 1994; Whaley et al. 1994), and the functional analysis of the altered VHL gene product will provide clarification of these issues.

Acknowledgements Supported in part by Deutsche Forschungsgemeinschaft (De 356/3-1), and Sander Foundation (93.038.1). The authors wish to thank Dr. Thomas Hankeln (Institute of Genetics, Johannes Gutenberg University Mainz, Germany) for the sequencing work; Dr. Helen Donis-Keller (Division of Human Molecular Genetics, Washington University, St. Louis, USA) and Dr. Harold Riethman (Wistar Institute, Philadelphia, USA) for providing the YAC clones; and Prof. Dr. Manfred Thelen (Institute of Radiology, Johannes Gutenberg University Mainz, Germany) for providing the magnetic resonance imaging and computed tomography scan.

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